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# Effect of Age and Route of Inoculation on Outcome of Neonatal Herpes Simplex Virus Infection in Guinea Pigs

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The morbidity and mortality of neonatal herpes simplex virus infection remains unacceptably high despite antiviral therapy. A better understanding of factors that might contribute to this poor outcome is needed but has been hindered by a lack of a good animal model. The recently described guinea pig model of neonatal HSV-2 infection was used to explore the effect of age and route of inoculation on the outcome of infection. After intranasal inoculation the onset, extent, and severity of the primary disease, as well as the number of recurrent lesion days, varied inversely with age. The route of inoculation also affected the outcome. Newborn animals were inoculated either intradermally on the scalp or by the intranasal, oral or corneal route. Animals inoculated on the scalp had the best outcome with no deaths or evidence of neurologic disease while the intranasal route produced the most severe disease, 88% mortality. Neurologic disease was common after oral (41%) and corneal (56%) inoculation but resolved spontaneously whereas following intranasal (39%) inoculation all animals with neurologic disease died. Recurrent disease manifest by cutaneous lesions was observed in all survivors of each group but also differed by the route of inoculation. The guinea pig model of neonatal HSV-2 disease appears to mimic human disease. The studies presented here show that the outcome of infection is influenced by the age and route of inoculation. 

**KEY WORDS:** neonatal HSV, pathogenesis, animal model, herpes simplex virus

## INTRODUCTION

Herpes simplex virus (HSV) infection is usually a self-limited disease in children and adults. However, in newborn infants it can be a devastating illness with unacceptable mortality and morbidity despite antiviral therapy [Whitley et al., 1980, 1983, 1988, 1991a,b]. Morbidity and mortality is least when disease is limited to the skin, eye, and mouth (SEM disease) and greatest with disseminated disease [Whitley et al., 1983, 1991a,b]. Isolated CNS disease is often associated with significant neurologic morbidity although mortality is less than that observed with disseminated disease [Whitley et al., 1991a].

In order to develop improved interventional strategies against neonatal HSV disease, it is important that the pathogenesis of this infection be better understood. Variables including age, site of inoculation, size of inoculum and viral strain differences may affect the outcome but are not well studied. Systematic study of the pathogenesis of this infection has been hampered by lack of a suitable animal model. Neonatal HSV disease in suckling mice is lethal, probably because of the severely compromised immune system in these animals, resulting in rapid dissemination of the infection [Kern et al., 1973]. Recurrent disease is seldom seen in survivors [Kern et al., 1973]. Newborn rabbits and hamsters have also been evaluated as experimental models because they are more immunologically mature at birth than the suckling mice. Unfortunately, the clinical disease in these animals did not mimic human illness [Kurata et al., 1976; Biegelisen and Scott, 1958].

Guinea pigs appear to be a promising model for the study of the pathogenesis of HSV disease. The pathogenesis of genital disease in adult animals has many similarities with human disease and is accepted by many as the animal of choice to investigate HSV infection [Stanberry et al., 1982; Hsiung et al., 1984; Stanberry, 1992]. Fur-

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TABLE I. Effect of Age at Inoculation on Outcome of HSV Infection in Guinea Pigs

| | | Type of disease | | | | |
|---|---|---|---|---|---|---|
| Age | N | SEM (%) | Resp. (%) | CNS (%) | Systemic (%) | Mortality (%) |
| 4–24 hours | 15 | 15 (100) | 15 (100) | 1 (6) | 15 (100) | 15 (100) |
| 36–48 hours | 15 | 15 (100) | 15 (100) | 4 (27) | 14 (93) | 11 (73) |
| 7 days | 15 | 15 (100) | 15 (100) | 3 (20) | 8*,** (53) | 7* (47) |
| Adults | 15 | 14 (93) | 12 (80) | 0 (0) | 4*** (27) | 2*** (13) |

*$P < 0.005$ vs. 4–24 hours.
**$P < 0.05$ vs. 36–48 hours.
***$P < 0.005$ vs. 4–24 hours or 36–48 hours.

ther, we recently described a model of HSV-2 infection in newborn guinea pigs [Bravo et al., 1994]. The outcome following intranasal inoculation was similar to that seen in human neonates, and recurrent disease was observed in most survivors. In evaluations presented here, we expanded our knowledge of this model by exploring the effect of age and route of inoculation on the outcome of HSV-2 infection.

## MATERIALS AND METHODS

### Cells and Virus

Rabbit kidney (RK) cells were prepared from 3-week-old *Pasteurella*-free, New Zealand white rabbits (Hazelton-Dutchland, Denver, PA) and maintained with Eagle's basal medium containing 2% fetal bovine serum with added antibiotics [Stanberry et al., 1982]. The HSV-2, MS strain (ATCC VR-540) was prepared in primary RK cells at a titer of $1.1 \times 10^7$ pfu/ml and stored frozen at −70°C.

### Animals

Timed pregnant Hartley guinea pigs (Camm Research, Wayne, NJ) were obtained 2 weeks prior to delivery (45–55 days gestation). Pregnant dams were evaluated twice daily at specific times until delivery. Adult Hartley guinea pigs >300 g (Charles River Laboratories, Wilmington, MA) were used as the control group.

### Experimental Design

In experiment 1, after delivery each litter was randomly assigned to be inoculated at either 4–24 hours (group 1), 36–48 hours (group 2), or 7 days (group 3) for a total of 15 animals per group. Only those animals with birth weights >50 g were selected. Adult guinea pigs served as controls (group 4) with one adult animal inoculated simultaneously with every five newborns. All animals were inoculated intranasally by swabbing both nostrils with a pre-moistened #4 Calgiswab prior to instillation of 15 μl of virus into each nostril 30 minutes apart. The total viral inoculum was $3.3 \times 10^5$ pfu.

In experiment 2, litter mates were randomized at delivery to one of the following sites of inoculation: intranasal (group 1), scalp (intradermal) (group 2), intraoral (group 3), or intracorneal (group 4). There were 18 animals per group except in group 3 (intraoral) which had 17 animals. Based on the results of the first experiment, all animals were inoculated at approximately 48 hours of age with $3.3 \times 10^5$ pfu of virus. Intranasal inoculation was performed as described above. Scalp inoculation was carried out by depilating the scalp of the animal and then scarifying the skin with a #25 G needle. Fifteen microliters of the virus was then applied to the scarified area twice, 30 minutes apart. For intraoral inoculation, the mucosa of both the upper and lower lips were scarified using a #25 G needle and viral inoculum applied as above. Intracorneal inoculation was performed as previously described [Wander et al., 1987]. Briefly, following local anesthesia with 0.5% proparacaine hydrochloride ophthalmic drops, the right cornea was trephined using 2–3 interlocking incisions approximately 3 mm in diameter and 0.5 mm in depth. Fifteen microliters of the virus was then instilled into the conjunctival sac twice, 30 minutes apart.

Newborn pups were maintained in their original litters along with their mothers until weaned. Animals were evaluated daily for signs of (1) cutaneous disease, including papules and vesicles; (2) respiratory disease, including tachypnea, nasal flaring and intercostal retractions; (3) CNS disease, including irritability, abnormal movements, seizures, paresis and paralysis; and (4) systemic signs, including lethargy, dehydration, weight loss (>10% of baseline body weight) and hypothermia (<37°C). All survivors were examined daily for recurrent disease from days 15–90 post-inoculation in experiment 1 and from days 15–45 in experiment 2.

In experiment 1, tissue samples were obtained for histopathology and viral isolation from liver, spleen, adrenals, lungs, midbrain, cortex, olfactory bulbs, facial and trigeminal nerves from pre-morbid or recently expired animals as previously described [Bravo et al., 1994].

## RESULTS

### Experiment 1

We evaluated initially the effect of age to establish the period of maximal susceptibility to intranasal inoculation. All but one animal developed clinical HSV-2 disease (Table I). The onset of clinical disease varied from 3–6 days with earlier onset in animals less than 48 hours of age. Cutaneous and/or ocular disease were observed in all symptomatic animals. Respiratory symptoms were observed in all animals inoculated at less than 7 days of life as compared to 80% of the adult animals. Neurologic disease was detected in only 6% of animals inoculated within 24 hours of birth due to rapid progression and

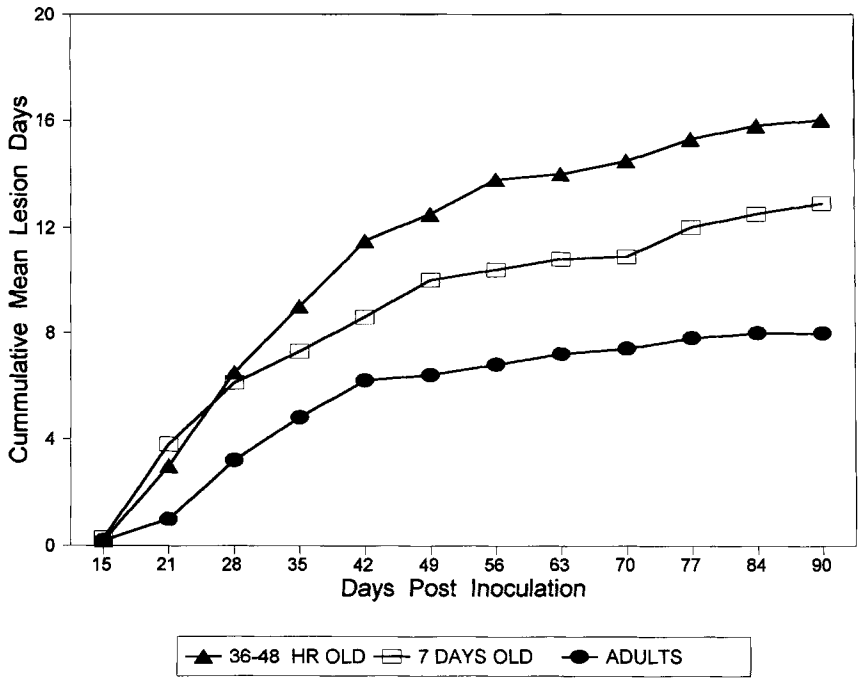


Fig. 1. Cumulative recurrent lesion days. Animals were followed daily for evidence of recurrent HSV disease from day 15 when the acute disease had resolved until day 90. Animals were inoculated either at 36–48 hours after birth, 7 days of age or as adults. There were no survivors in those inoculated at 4–24 hours after birth.

early death. This increased to 27 and 20% of pups inoculated at 36–48 hours and 7 days, respectively, but was not seen in adult animals.

Mortality and systemic symptoms appeared to be age-related (Table I). Systemic symptoms were detected in 100, 93, 53, and 27% of groups 1–4 respectively, while mortality decreased from 100% of animals inoculated at 4–24 hours of age to 13% of adults ($P < .005$). A significantly higher mortality was observed comparing animals inoculated at 4–24 hours to those inoculated at 7 days.

All survivors developed spontaneous recurrent disease between days 15–90 as evidenced by the presence of single or multiple papular and/or vesicular lesions. Animals inoculated at 36–48 hours developed more recurrent lesion days ($16.0 \pm 4.6$) compared to either those inoculated at 7 days ($12.9 \pm 3.2$, N.S.) or adults ($8.0 \pm 2.6$, $P < 0.02$) (Fig. 1).

HSV was recovered from the tissues of several morbid and pre-morbid animals as shown in Figure 2. The viral recovery rate was higher from neural tissues as compared to the extra-neural sites. Because virus was recovered from the lungs infrequently, it appears that death occurred predominantly because of CNS involvement.

### Experiment 2

In order to compare different routes of inoculation, all animals were given the same dose at 48 hours of age in this experiment. Clinical manifestations of both primary and recurrent infections varied by the route of inoculation. As shown in Table II, all but one animal (70/71) developed cutaneous disease mostly at and around the site of inoculation. Respiratory symptoms were present predominantly in the intranasal group (18/18) while the incidence of neurologic disease was lowest in the intradermal group (0/18), but appeared to be similar in the intracorneal (10/18), intranasal (7/18), and intraoral groups (7/17). Mortality was observed between days 6–9 and was highest in the intranasal group (89%) compared to the other groups (0–6%) ($P < 0.001$). CNS disease resolved spontaneously between days 15–30 in most of the animals infected by the intracorneal or intraoral routes, but resolution was unusual in the intranasal group where mortality was high.

Recurrent disease was observed in all survivors with symptomatic disease (Fig. 3). Recurrences were higher in the intracorneal group compared to the intradermal ($P = 0.002$) and intraoral groups ($P = 0.07$). There were too few survivors in the intranasal group (N = 2) for meaningful comparison of their recurrence rate, but recurrences were frequent in the two survivors.

## DISCUSSION

The incidence of neonatal herpes simplex virus infections appears to be increasing as genital herpes becomes more common among adolescents and young adults in the United States [Stone et al., 1989; Sullivan-Bolyai et al., 1983]. Untreated HSV infection in newborn infants has a high mortality rate and, despite the availability of appropriate antiviral therapy, neonatal HSV infection continues to have unacceptable morbidity, especially in those with disseminated or CNS disease [Whitley et al.,

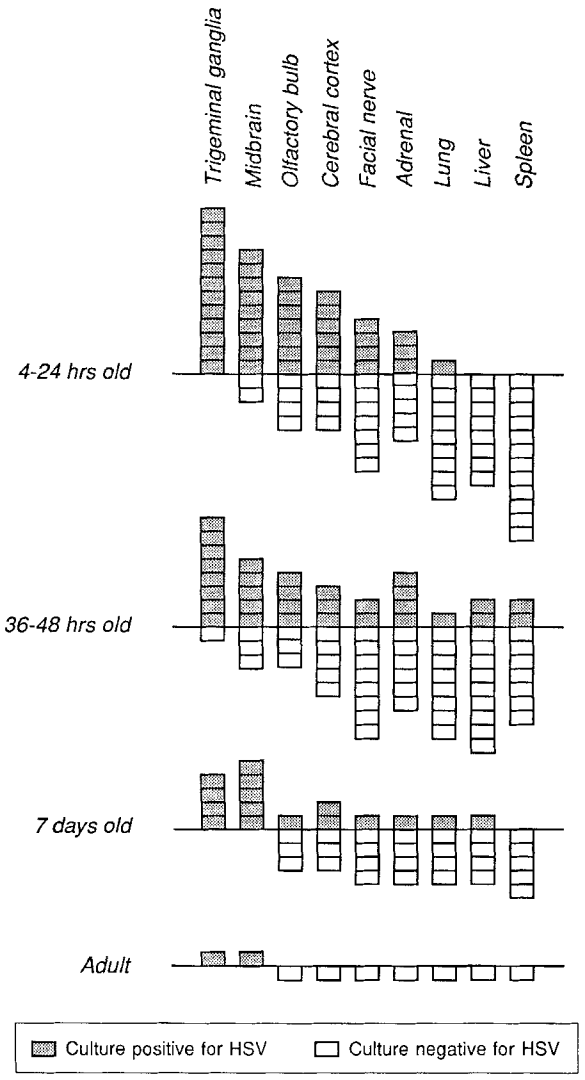


Fig. 2. Effect of age on HSV-2 virus dissemination. Tissues from morbid and premorbid animals infected intranasally with HSV-2 were homogenized and cultured for evidence of HSV-2 infection. Those above the line were culture positive and those below culture negative.

1983, 1991a,b]. Several animal models of neonatal HSV disease have been developed in the past in an attempt to study the pathogenesis of this disease [Kern et al., 1973; Kurata et al., 1976; Biegelisen and Scott, 1958]. Most of these models, while useful in therapeutic trials, have been of limited value in understanding the pathogenesis of human neonatal disease. The suckling mouse model has a lethal outcome while the newborn rabbits and hamsters demonstrate clinical HSV disease that is different from that observed in the human newborns.

The guinea pig has been shown to be a useful model for the study of pathogenesis of human genital HSV infections [Stanberry, 1992; Stanberry et al., 1982; Hsiung et al., 1984] but is less well studied as a model of neonatal HSV infection. In a previous study using newborn guinea pigs, 30% of animals developed fatal encephalitis after intracorneal inoculation with HSV-1 as compared to only 1–2% of the adult animals [Tenser and Hsiung, 1977]. Latent HSV was also detected more frequently in the trigeminal ganglia of newborns compared to adult animals. Following intranasal inoculation of HSV-2 within 24 hours of birth, we previously showed that newborn guinea pigs developed both primary and recurrent HSV-2 disease that was modified by treatment with intraperitoneal acyclovir [Bravo et al., 1994]. In studies presented here, we describe the effect of age and portal of entry on the outcome of HSV-2 infection in newborn guinea pigs. In these experiments, we noted that the onset, extent and severity of neonatal HSV-2 infection varied inversely with the age of the animal. Likewise, recurrent disease was more frequent in the animals inoculated before 48 hours of age. Recurrent disease is also a frequent problem in neonatally infected infants where the number of recurrences has been correlated to outcome [Whitley et al., 1991a]. In this report we also note that the outcome of infection in animals inoculated before 24 hours and between 24–48 hours were similar, thus extending the utility of this model by eliminating the need for constant monitoring for delivery.

Immature immune mechanisms compounded with inadequately developed anatomical barriers probably play a significant role in the overall poor outcome observed in the very young animals. Newborn guinea pigs appear to exhibit some of the same immunologic defects that contribute to the susceptibility of human neonates to HSV infection [Wilson, 1990; Nair et al., 1985; Shore et al., 1977]. Thus, the effector function of leukocytes from newborn guinea pigs in antibody dependent cellular cytotoxicity assays (ADCC) is about half of that of an adult [Bravo et al., 1995] while a deficiency in non-specific killing of HSV-infected targets has also been described [Fowler et al., 1992].

Clinical manifestations of primary HSV-2 infection also varied with the route of inoculation. Overall, 70/71 inoculated animals showed clinical evidence of primary HSV-2 disease regardless of route. Animals inoculated intradermally on the scalp, however, had the mildest disease and the best outcome. Their disease was limited to the skin at the site of inoculation. None of these animals died as a consequence of primary HSV infection, correlating with similar clinical observation in human neonates with primary localized cutaneous HSV disease [Whitley et al., 1991a]. Further, none of the animals developed CNS disease during the primary illness. This is quite different from the clinical observation of infants where the majority of untreated human neonates with localized cutaneous disease ultimately progress to disseminated and/or neurological disease [Whitley et al., 1980].

Progression to neurologic disease, evidenced by paralysis of one or more limbs, however, was observed in 56%, 41% and 39% of the intracorneal, intraoral and intranasal groups, respectively. Seizures or encephalopathic symptoms were less often observed. The severity of neurologic disease appeared to differ between the involved groups. All the intranasally inoculated animals

TABLE II. Outcome of Neonatal HSV-2 Infection in Guinea Pigs Inoculated by Four Different Routes

| Group | N | Type of disease | | | | |
|---|---|---|---|---|---|---|
| | | SEM (%) | Resp. (%) | CNS (%) | Systemic (%) | Mortality (%) |
| Intranasal | 18 | 18 (100) | 18 (100)* | 7 (39) | 17 (94)* | 16 (89)* |
| Scalp | 18 | 18 (100) | 0 (0) | 0 (0)** | 0 (0) | 0 (0) |
| Intraoral | 17 | 17 (100) | 1 (6) | 7 (41) | 2 (12) | 1 (6) |
| Intracorneal | 18 | 17 (94) | 0 (0) | 10 (56) | 1 (5) | 1 (6) |

*$P < .001$ vs. other groups.
**$P < .005$ vs. other groups.

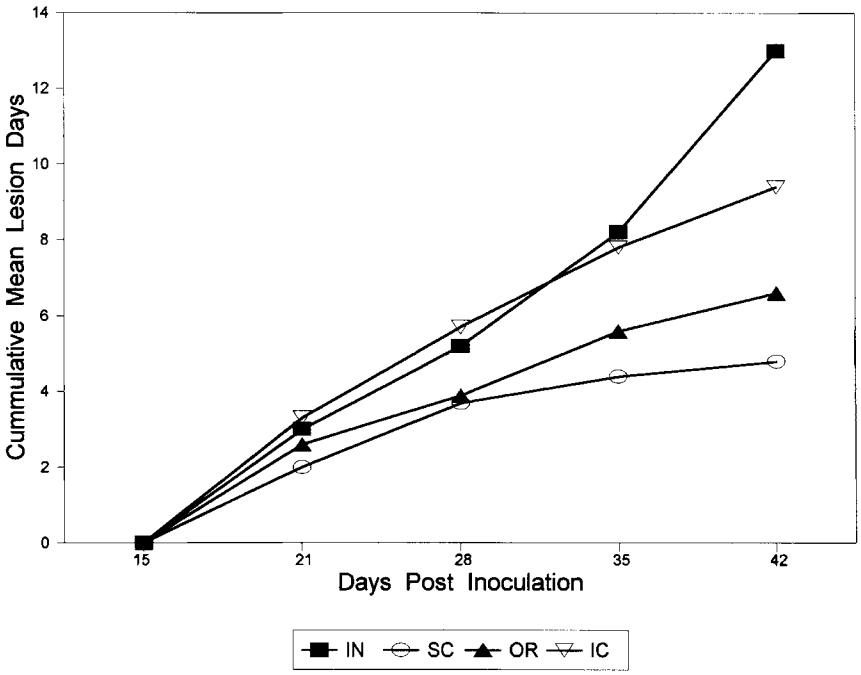


Fig. 3. Cumulative recurrent lesion days. Animals were followed daily for evidence of recurrent HSV disease from days 15–42. Animals were inoculated either intranasally (IN), on the scalp (SC), intraorally (OR), or intracorneally (IC). Each group represents the mean of at least 16 animals except the intranasal group where only two animals survived.

with neurologic disease had a fatal outcome which is consistent with previous studies showing that intranasal inoculation leads rapidly to CNS involvement [Tenser and Hsiung, 1977; McLean et al., 1989]. Animals in the intracorneal group experienced moderate neurologic involvement, but none became encephalopathic as described in the article by Tenser and Hsiung [1977], and only one died. This may be due to strain and HSV type differences between the two experiments. Animals in the intraoral group developed neurologic disease at about the same rate as the intracorneal group and also recovered. In most survivors, gross neurologic signs resolved spontaneously within a month. No apparent difference in the rate of recovery was observed between the different groups. It would appear most likely that differences in the magnitude and areas of CNS involvement following the different routes of inoculation account for these differences in outcome.

Overall outcome was worst in the intranasal group with the highest morbidity and mortality (>88%). Respiratory illness was predominant in this group leading us to speculate that most of these animals might have had HSV pneumonia or aspiration pneumonitis. However, we were unable to recover the virus from the lungs of most morbid animals. Viral recovery was, however, greatest in these animals from neural sites, especially from the olfactory bulb, trigeminal ganglion and midbrain, thus leading us to hypothesize that the most probable cause of increased morbidity and mortality in this group was the rapid intraneural spread to the brain via the richly innervated cribriform plate in the roof of the nose with subsequent development of fulminant encephalitis, although aspiration pneumonitis might have been a complication of encephalitis.

In summary, the outcome of both primary and recurrent HSV infections in newborn guinea pigs appears to

be influenced by the age at and route of inoculation. The clinical syndrome observed in these animals was analogous to that seen in human neonatal HSV infection. Further studies may be necessary to define the age and site dependent factors that modify the outcome from this disease. This model should prove useful in defining further the pathogenesis of neonatal HSV disease.

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